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The Significance of Glutathione Conjugation in Aflatoxin Metabolism

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1. Introduction

We all are exposed through air, food, drinks and skin contacts to harmful compounds throughout the period of our lifetime, including, a variety of pharmaceuticals and food-derived carcinogen metabolite (e.g. N-acetoxy-PhIP), [52], plant toxins (such as *glycoalkaloids* in nightshades¹, *cyanogenic glucosides*², or *pyrrolizidine alkaloids* in some herbs and herbal teas), xenobiotics³ producing during early human pregnancy, fungal and bacterial toxins such as aflatoxins⁴; and cyanotoxin⁵; as well as free radicals and hydroperoxides. Many of these compounds are lipophilic and the organism can get rid of them only through metabolism.

Biotransformation has been conveniently categorized into three distinct phases, which act in a tightly integrated manner. Phases I and II enzymes catalyze the conversion of a lipophilic, non-polar xenobiotic into a more water-soluble and therefore less toxic metabolite, which can then be more easily excreted from the body. Phase I biotransformation seems to be enzymes that catalyzes oxidation, reduction or hydrolyze reactions, it usually converts substrates to more polar forms by introducing or unmasking a functional group (e.g., –OH, –NH₂, or –SH). Phase I consist primarily of microsomal enzymes, which are found abundantly in the liver, gastrointestinal tract, lung and kidney, consisting of families and subfamilies of enzymes that are classified based on their amino acid sequence identities or similarities. [84]. Many of the enzymes like monooxygenases are found in the endoplasmic

1 - Plants like potatoes, tomatoes, peppers, egg plant, tobacco, some spices.

2- Like bitter almond, cassava root, sorghum root, lima bean, fruit seed, etc.

3 - Chemical compounds foreign to the human organism without nutritional value

4 - A group of mycotoxins of which aflatoxin B1 is the most potent hepatocarcinogen

5 - A toxin producing by cyanobacteria of which microcystin-LR is predominant

reticulum membrane, but others such as the dehydrogenases for example alcohol dehydrogenases and peroxidases located in the cytoplasm, while still others such as monoamine oxidase are localized in mitochondria. Monooxygenases are also known as mixed function oxidases because in a typical reaction, one molecule of oxygen is consumed (reduced) per substrate molecule: one oxygen atom appearing in the product and the other in a molecule of water. The reaction scope of monooxygenases includes heteroatom oxidation, aromatic and aliphatic hydroxylation, epoxidation, and Baeyer-Villiger oxidation. There are two major types of microsomal monooxygenase, both of which require NADPH as an external reductant: the cytochrome P450 (CYP) system and flavin-containing monooxygenases. The mechanism of CYP is a complex cascade of individual steps involving the interaction of protein redox partners and consumption of reducing equivalents, usually in the form of NADPH. The iron heme containing enzyme, CYP, consists of two enzymes: NADPH-cytochrome P450 reductase and CYP. It is involved in the oxidative metabolism of many endogenous substances such as steroids and bile acids, as well as the detoxication of a wide variety of xenobiotics. It can oxidize AFB₁ to several products. Only one of these, the 8,9-exo-epoxide, appears to be mutagenic and the others are detoxification products. P4503A4, which can both activate and detoxicate AFB₁, is found in the liver and the small intestine. [33], [52]. Flavincontaining monooxygenases catalyze an NADPH- and an oxygen-requiring oxidation of substances (primarily xenobiotics) bearing functional groups containing nitrogen, sulfur, or phosphorus. The properties of the CYPs electron transport systems have also been reported [77].

In detoxification pathway, a series of enzyme-catalyzed processes with broad specificities convert the toxic substances into less toxic metabolites by chemical reactions within the body. Although biotransformation reactions take place within cytoplasm and mitochondria but they mostly happen within endoplasmic reticulum (E.R). Cell types also differ in their biotransforming potential for example cells located near the major points of xenobiotic entry into the body such as liver, lung, and intestine possess greater concentrations of biotransforming enzymes than others [52].

Phase II conjugation reactions which generally act follow phase I activation consists of reactions in which metabolites containing appropriate functional groups are conjugated with substances such as glucuronate, glutamate, sulfate, reduced glutathione or uridine diphosphate (UDP)-glucuronic acid to finally discharge them through urine or bile. In general, conjugation dramatically improves solubility, which then promotes rapid excretion. Among the several types of conjugation reactions which are present in the body, including glucuronidation, sulfation, and glutathione and amino acid conjugation, glutathione which is catalyzed by glutathione S-transferases, is the major phase II reaction in many species [52]. With the exception of acetylation, methylation and fatty acid conjugation, the strategy of phase II biotransformation is to convert a xenobiotic to a more hydrophilic form via the attachment of a chemical moiety which is ionizable at physiological pH. This metabolic transformation also results in reduced affinity of the compound for its cellular target. [67], [23].

In animals, elimination of the soluble compounds from cells and excretion of biotransformed molecules from the body referred to as phase III. It has been suggested that the

phase III of detoxification system to be called antiporter activity. Antiporter activity is an important factor in the first pass metabolism of pharmaceuticals and other xenobiotics. The antiporter is an energy-dependent efflux pump, which pumps xenobiotics out of a cell, thereby decreasing the intracellular concentration of xenobiotics. In eukaryotic organisms, they are actively excreted or compartmentalized in the vacuole by ATP-dependent GS-X pumps [42], [27]. Indeed, as the glutathionylated moiety is hydrophilic, the conjugate cannot usually simply re-diffuse back into the cell [77]. Antiporter activity in the intestine appears to be co-regulated with intestinal phase I CYP3A4 enzyme. This observation suggests the antiporter may support and promote detoxification. Possibly, its function of pumping non-metabolized xenobiotics out of the cell and back into the intestinal lumen, may allow more opportunities for phase I activity to metabolize the xenobiotic before it is taken into circulation. Although, most literature on detoxification refers to liver enzymes, as the liver is the site of the majority of detoxification activity for both endogenous and exogenous compounds, however, the first contact the body with the majority of xenobiotics take places in the gastrointestinal tract. Intestinal mucosa possesses enzyme systems capable of various types of biotransformation of xenobiotics [52]. Among the detoxification pathways, glutathione conjugation pathway is the prominent route of AFB1 inactivation in liver of mammals. Depending on the availability of cellular GSH and the activation of glutathione S-transferase subclasses, detoxification of AFB1 is facilitated [24].

2. Glutathione

Glutathione is a ubiquitous thiol-containing isotriptide (γ -glu-cys-gly, FW 307.3), consisting of glycine, glutamic acid and cysteine molecules which was first discovered by Sir Frederick Gowland Hopkins in 1920s, synthesized de novo in mammalian cells (Figure 1). This water soluble antioxidant compound is an unusual peptide in that the peptide bond between the glutamate residue and the cysteine residue is formed with the γ -carboxylate group of the former rather than the α -carboxylate group. Today along with β -carotene, ascorbic acid (vitamin C), α -tocopherol (vitamin E) and flavonoids *etc.*, GSH⁶ is commonly referred to as an antioxidant [17], which neutralizes free radicals due to the high electron-donating capacity of its sulfhydryl (-SH) group, [13], and prevents damage to important cellular components, implicates in the cellular defense against xenobiotics. Glutathione status is a highly sensitive indicator of cell functionality and viability. Its levels in human tissues normally range from 0.1 to 10 mM, being most focused in liver (up to 10 mM) and in the spleen, kidney, lens, erythrocytes and leukocytes and its emptying be joined to a variety of diseases. Under normal conditions, glutathione is predominantly present in its reduced form, with only a small proportion present in its fully oxidized state [20].

Moreover, the GSH/GSSG⁷ pair with their high reduction potential participates in maintaining other cellular thiol in a reduced state. Finally, GSH tends to a substrate or cofactor in some of

6 - Glutathione, reduced form

7 - Glutathione, oxidized state

GSH linked enzymes. There are a number of GSH linked enzymes that are involved in cellular protection against toxic substances. The glyoxalase I and II which are responsible for catalyzing the conversion of methylglyoxal (a by-product in glycolysis) to lactic acid are among these enzymes [76]. Glutathione reductase (GR) which catalyzes the reduction of GSSG using NADPH as a reductant is also a glutathione-linked enzyme involved in cell protection. GR is important to keep the high cellular reductive potential. Selenium dependent glutathione peroxidase are other GSH-linked enzymes that catalyze the reduction of peroxides using GSH as the reducing agent [7]. Finally, last but not the least, glutathione transferases are also GSH dependent enzymes with many properties among which catalyzing the conjugation of GSH to various electrophilic compounds is one of the most investigated function [25].

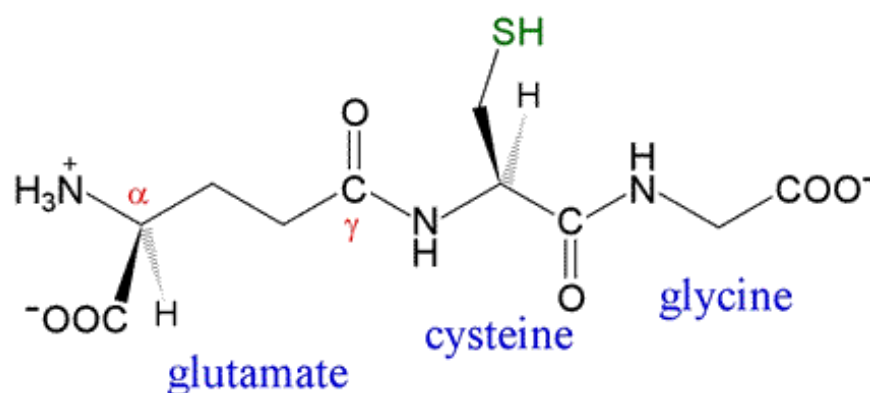


Figure 1. Structure of reduced glutathione; glutamate is linked in an isopeptide bond (via its γ -carboxyl group) to cysteine, which in turn forms a peptide linkage with glycine

3. Glutathione S-transferase

Glutathione S-transferases (GST, EC 2.5.1.18), which first discovered as enzymes in 1961 [12], are abundant proteins encoded by a highly divergent, ancient gene family. These major cellular detoxification enzymes present mostly in liver and kidney as well as intestine. In spite of 40 years of research the exact function of this protein is more complex than ever, but it has been found that these intracellular dimeric proteins, play a major role in the intracellular transport of endogenous compounds, metabolizes various electrophilic xenobiotics, ligand transport and thus protects cells against toxic effects [31], [87], [85]. GST catalyzes the conjugation of glutathione on the sulfur atom of cysteine to various electrophiles and catalyses the conjugation of various electrophiles with GSH, detoxifying both exogenously and endogenously derived toxic compounds [13].

3.1. Classification and structure

The superfamily of the glutathione transferases are divided into at least four major families of proteins, namely cytosolic or soluble GSTs, mitochondrial GSTs, microsomal GSTs and

bacterial fosfomycin-resistance proteins [39], [6], [69]. The cytosolic GSTs (cGSTs) have been subgrouped into numerous divergent classes on the basis of their chemical, physical and structural properties [39], [70]. The mitochondrial GSTs, also known as kappa class GSTs, are soluble enzymes that have been characterized in eukaryotes [65]. The third GST family comprises membrane-bound transferases called membrane-associated proteins involved in eicosanoid and glutathione metabolism, but these bear no similarity to soluble GSTs [44]. Representatives of all three families are also present in prokaryotes but the fourth family is found exclusively in bacteria [4]. The mammalian soluble GSTs are so far divided into eight classes based on their amino acid sequences including: Alpha (α), Kappa (κ), Mu (μ), Omega (ω), Pi (π), Sigma (σ), Theta (θ) and Zeta (ζ), [11], [78], [64], [40]. GSTs are named using a letter corresponding to their class membership and Arabic numerals after the subunit composition (*e.g.* GST A1-1 is a homodimeric alpha class GST consisting of two subunit 1).

3.2. Presence of GST in cells

3.2.1. Microbial GST

For a long time, GST enzymes from microbial sources were neglected and were not systematically studied. One of the reasons for this was the poor activity of microbial GSTs with CDNB⁸ as a model substrate for GST activity, which led to the conclusion that these enzymes are rare in unicellular organisms [81], [77]. The first evidence for the presence of GSTs in bacteria was reported more 30 years ago by Takashi Shishido who showed the presence of GST activity in a strain of *Escherichia coli* [71]. Since then, GSTs have been found to be broadly distributed in aerobic prokaryotes, but not in anaerobic bacteria [59]. The absence of the enzyme in these microorganisms is consistent with the lack of GSH [28]. Bacterial glutathione transferases are part of a superfamily of enzymes that play a key role in cellular detoxification. Bacterial GSTs are implicated in a variety of distinct processes such as the biodegradation of xenobiotics, protection against chemical and oxidative stresses and antimicrobial drug resistance. In addition to their role in detoxification, bacterial GSTs are also involved in a variety of distinct metabolic processes such as the biotransformation of dichloromethane, the degradation of lignin and atrazine, and the reductive dechlorination of pentachlorophenol [4], [51].

3.2.2. GSTs of fungi and yeasts

Until recently, relatively little was known about the presence and role of GST in fungi. However, expression of GST has been reported in some fungal species such as *Issatchenkia orientalis*, [73], *Phanerochaete chrysosporium*, *Yarrowia lipolytica*, *Mucor circinelloides* [70] *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus fumigatus* [Burns et al., 2005] *Saccharomyces cerevisiae*, and *Cunninghamella elegans*, [70] [48] *etc.* However, the role of the enzyme in fungi, particularly toxigenic strains, is not well understood [2]. Although it has been shown that GST has a significant role in detoxifi-

8 - 1-chloro-2,4-dinitrobenzene

cation of aflatoxin and there is a possibility that this enzyme catalyses the conjugation of GSH to AFB₁-epoxide to excrete its derivatives from the body, in 1988 and for the first time Saxena et al. reported that the relation of cytosolic GSH S-transferases from *A. flavus* to aflatoxin synthesis. In truth, they showed that in contrast to other cells that GST has a critical function to break down the aflatoxin, in aflatoxigenic *Aspergillus* spp., there is positive correlation between the GST activity and aflatoxin production [68], factors influencing aflatoxin formation such as growth period, medium etc., always enhanced GST activity in the toxigenic strain. Since the non-toxigenic strain produces no aflatoxin, these factors have little effect on its GST activity. Our experience with GSH-conjugation system using inducers/inhibitors of aflatoxin metabolism in fungi also show a positive correlation of aflatoxin synthesis and GST activity in *Aspergillus* species [2], [88].

3.2.3. Plant GSTs

Plant GSTs are a family of multifunctional enzymes involved in the intracellular detoxification of xenobiotics and toxic compounds produced endogenously [54], [26]. Most of the enzymes are stress-inducible and play a role in the protection of plants from adverse effects of stresses. However, the activities of different GSTs have been detected and characterized in many plants, including maize, wheat, tobacco, soybean, barley, chickpea, peanut, sorghum, and sugarcane [20], [21], [22], [75].

3.2.4. Mammalian GST

The isoenzymes of glutathione transferase have been most widely studied in rat liver. Six basic transferases in rat liver have been characterized. In rabbit, GST catalyzes the conjugation of activated AFB₁ with glutathione. In an experiment to assess the abilities of lung and liver GSTs to conjugate AFB₁-8, 9-epoxide, it has been shown that alpha-class and mu-class GSTs are of similar importance in catalyzing the reaction in the lung. The human glutathione S-transferase, possess both enzymatic and non-enzymatic functions and are involved in many important cellular processes, such as, phase II metabolism, stress response, cell proliferation, apoptosis, oncogenesis, tumor progression and drug resistance. The nonenzymatic functions of GSTs involve their interactions with cellular proteins, such as, Jun N-terminal kinase (JNK), tumor necrosis factor receptor-associated factor-2 (TRAF2), apoptosis-signal-regulating kinase 1 (ASK), serine/threonine kinases (PKA, PKC), and tissue transglutaminase 2 (TGM2), during which, either the interacting protein partner undergoes functional alteration or the GST protein itself is post-translationally modified and/or functionally altered [53], [74].

3.3. Different functions of GST

3.3.1. The metabolic function of GSTs

GSTs have been reported to involve in steroid metabolism by catalyzing the isomerization of Δ^5 -androstene-3, 17-dione to Δ^4 -androstene-3, 17-dione, and biosynthesis of prostaglandins. GST M2-2 is a prostaglandin E synthase in the brain cortex [8] and rat GST A1-1 and GST

A3-3 catalyze the reduction of PGH₂ to PGF₂. The isomerization reaction of PGF₂ to PGD₂ is also catalyzed by sigma class of GST. PGD₂, PGE₂ and PGF₂ act as hormones that bind to G-protein coupled receptors which regulate other hormones and neurotransmitters. Prostaglandin D₂ and E₂ are unstable and will easily be converted to prostaglandin J₂ and A₂, respectively and their derivatives inhibit NFκB, [66] a family of transcription factors that regulate the transcription of genes important for inflammatory processes. There are interesting speculations that GSTs might block other anti-inflammatory pathways by catalyzing the conjugation of GST to PGJ₂ [38], [25]

3.3.2. *The ligandin function of GSTs*

Because of exhibiting a ligand binding function, glutathione transferases, have been known as ligandin, a function, which involves the noncovalent binding of nonsubstrate hydrophobic ligands such as heme, bilirubin, various steroids, and conceivably some lipophilic anti-cancer drugs as well. Although GSTs are generally viewed as playing a protective role in foreign compound metabolism, they can also catalyze reactions that lead to toxification. Examples include the GST dependent metabolism of 1,2-dibromoethane and related haloalkanes and probably also metabolism of the 6-thiopurine prodrug azathioprin [60], [5]. Similarly, the cytotoxicity of the polypeptide antibiotic neocarzinostatin is greatly enhanced by thiols such as GSH, although in this case there is no apparent requirement for GST catalysis [25], [18]. [82].

3.3.3. *The regulatory function of GSTs*

In addition to above functions, GSTs also are responsible for interacting the proteins and enzymes. For example GST P1-1 interacts with c-Jun N-terminal kinase 1 (JNK1) suppressing the basal kinase activity. GST P1-1 also has a role in protection and cell survival after exposure to H₂O₂ but not against UV-induced apoptosis [1]. Whereas, mouse GST M1-1 protects cells against both UV- and H₂O₂-induced cell death and binds to apoptosis signal-regulating kinase 1 (ASK1), inhibits its kinase activity [16]. Moreover, mouse GST A4-4 has also been proposed to interact with JNK and prevent cells from 4-hydroxynonenal induced apoptosis [15], [25].

3.3.4. *The detoxification function of GSTs*

As enzymes, GSTs are involved in many different detoxification reactions. They are commonly referred to as phase II enzymes. They catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic toxic compounds. The GSH conjugates are excreted as mercapturic acids by the phase III metabolic pathway [41]. GST P1-1, GST M1-1 and GST A1-1 have been shown to catalyze the inactivation process of α, β unsaturated carbonyls like acrolein, (a cytotoxic compound present in tobacco smoke), propenals, (generated by oxidative damage to DNA) and alkenals, (formed by oxidative damage to lipids) [25], [70].

3.4. GST and aflatoxin

3.4.1. Introduction

Study on GSTs of *Aspergillus flavus* stems from its ability to synthesize the aflatoxin. Aflatoxins are one of the major causes of liver cancer in certain regions of Africa and Asia [83], [61]. These secondary metabolites which primarily produced by some *Aspergillus* spp. are ubiquitous, and under favorable conditions can grow on a wide variety of agricultural commodities. Aflatoxins are major concern with to public health and the most important toxicological interest in aflatoxins has concentrated on aflatoxin B₁, largely due to its acute toxicity and carcinogenicity in humans and animals. [3], [62], [88]. Genetic studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* has been led to the cloning of 25 clustered genes within a 70 kb DNA region responsible for the enzymatic conversions in the aflatoxin biosynthetic pathway [86].

3.4.2. Primary metabolism of aflatoxin B₁

Once inside the body and for toxicity to occur, AFB₁ undergoes enzymatic conversion to electrophilic endo and exo stereoisomers of AFB₁-8,9-epoxide by the action of mixed function mono-oxygenase enzyme systems, CYPs are an intensively studied family of enzymes with currently approximately 4,000 known members. They have been found in almost all branches of the "tree of life", ranging from microorganisms over plants to mammals. CYP enzymes are classified into families identified by a number (e.g., 1, 2, 3, and 4), subfamilies identified by a letter (e.g., 2A, 2B, 2D, and 2E), and specific members identified by another number (e.g., CYP2E1 and CYP2A6) [47], [19].

In human, five CYP gene families, namely; CYP1, CYP2, CYP3, CYP4 and CYP7 are believed to play crucial roles in hepatic as well as extra-hepatic metabolism and elimination of xenobiotics [50], [58], [84]. This superfamily of hemoproteins aids in the oxidation of various substrates such as steroids, eicosanoids, pharmaceuticals, pesticides, pollutants, and carcinogens [57]. As mentioned earlier, they bioactivate AFB₁ to an electrophilic, highly reactive and unstable metabolite known as aflatoxin-8,9-epoxide, which binds to guanine residues in nucleic acids, leading to irreversible damage in DNA and causing hepatocarcinoma in humans, primates, and ducks [32], [84]. However, only AFB₁ exo-epoxide (AFBO), binds appreciably to DNA (Figure 2). The AFBO is highly unstable, and it reacts with cellular nucleophiles and can induce mutations by alkylating DNA, principally at the N⁷ position of guanine forming the 8,9-dihydro- 8-(N⁷-guanyl)-9-hydroxy-AFB₁. In addition, AFBO can bind to proteins and other critical cellular nucleophiles [43], [63]. Initial studies reported that concentrations of AFB₁ which are likely to be achieved in the liver following ingestion of "real-world" concentrations of AFB₁ are bioactivated to AFBO primarily by CYP1A2, whereas much higher concentrations are catalyzed by CYP3A4 [30], [46], [79]. A recent study demonstrated a dominant contribution of CYP3A4 homologues in AFBO production. AFB₁ metabolism studies in human liver microsomal preparations indicate a predominant role for CYP3A4 and that its expression level was an important determinant of the AFB₁ disposition in human liver [45]. Specific CYP3A4 inhibitors like troleandomycin have been shown to in-

hibit AFBO production [29], while inducers of CYP3A4 activity such as 3-methylcholanthrene and rifampicin, increase AFB₁ metabolism in cultured human hepatocytes [49].

CYP1A homologues also metabolize AFB₁ to produce the detoxified metabolite AFM₁, whereas CYP3A enzymes⁹, produce another detoxified metabolite, aflatoxin Q₁ (AFQ₁), the major metabolite of AFB₁ (Figure 2). [33]. Although both CYP1A and CYP3A isoforms oxidize AFB₁, there are conflicting reports on their relative importance [63].

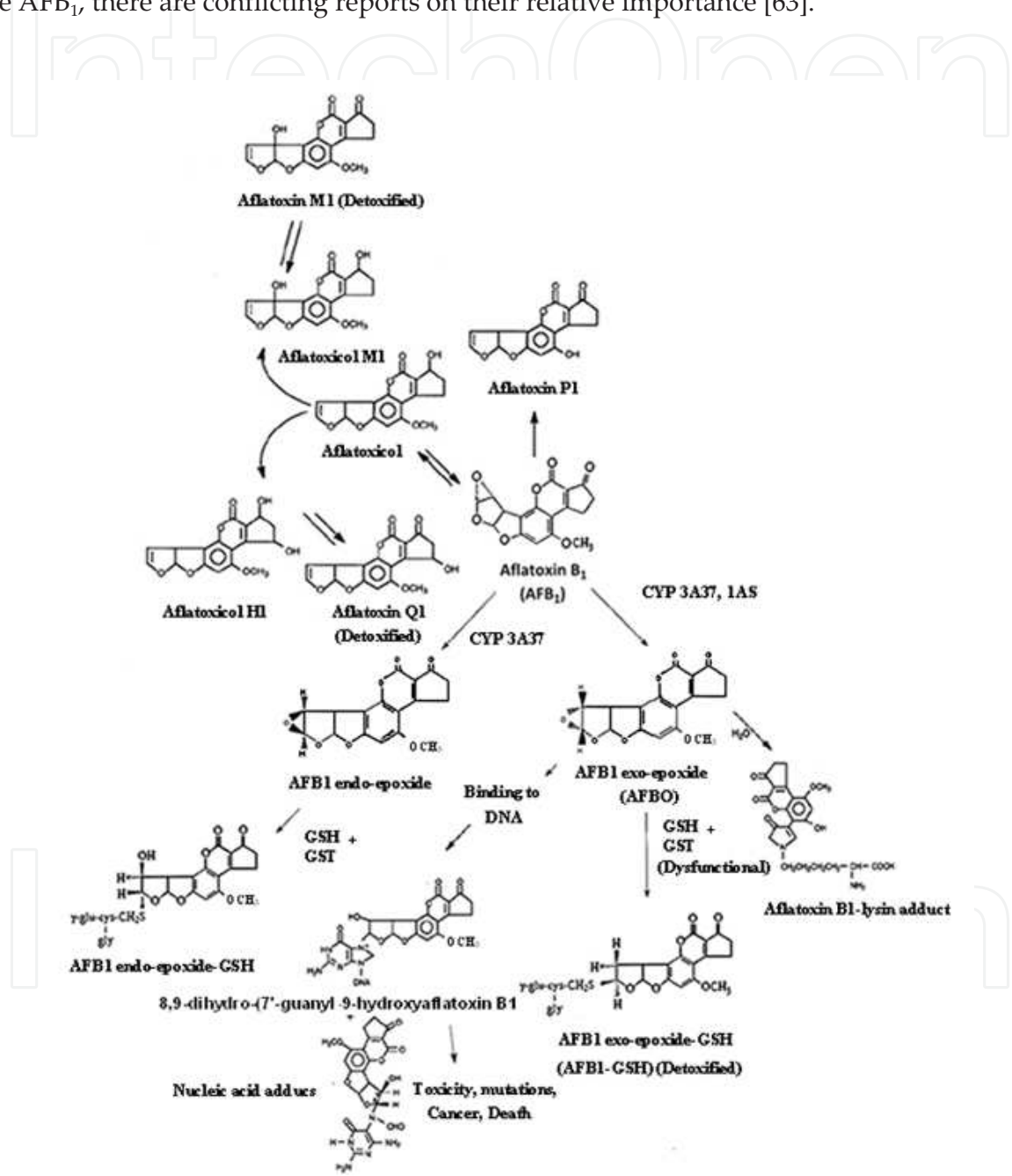


Figure 2. Bioactivation of AFB₁ to exo and endo-epoxides and subsequent GST-catalyzed conjugation with GSH.

CYPs may also catalyze demethylation to aflatoxin P (AFP₁) of the parent AFB₁ molecule, resulting in products less toxic than AFB₁. Other major metabolites in the human include aflatoxicol (AFL), AFLH₁, AFB₂á and AFB₁-2, 2-dihydrodiol [80].

3.4.3. Secondary metabolism of aflatoxin B₁

Oxidative metabolism of AFB₁ by cytochrome P450 results in the formation of several products such as AFB₁-epoxide which serve as substrates for phase II detoxification enzymes. Phase II enzymes such as GSTP1 and GSTA1, found in several mammalian species and non-tumorous liver tissues [14] are the first step in the mercapturic acid pathway, which leads to the excretion of the xenobiotics. Because conjugation of the electrophilic AFB₁-8,9-epoxide with GSH is an alternative fate to binding to nucleophilic centers in cellular macromolecules, GSTs play a critical role in the protection of tissues from the deleterious effects of bio-activated AFB₁, and tissues vary considerably in both GST concentration and distribution of specific GST isoforms. Two stereoisomers of AFB₁-8,9-epoxide were identified: AFB₁ exo-epoxide and AFB₁ endo-epoxide, and their corresponding GSH conjugates; AFB₁ exo-epoxide-GSH and AFB₁ endo-epoxide-GSH. It has been reported that only the exo-epoxide effectively interacts with DNA and was at least 500-fold more potent as a mutagen than the endo stereoisomer. [43], [72].

Throughout the animal kingdom, significant variations exist in the susceptibility of different species to AFB₁. Man and rats are sensitive to AFB₁ but mice can tolerate this mycotoxin. [35]. In man and rat as well as many mammalian species, AFB₁-8,9-epoxide is efficiently conjugated with reduced glutathione. Little is known about the identity of the GST which is responsible for detoxifying activated AFB₁. To date, the catalytic conjugation of AFB₁-8,9-epoxide has only been reported using rat and mouse GST as enzyme source and the ability of GST in other species to catalyze this reaction has not been described. In the investigation on hepatic rat GST responsible for catalyzing the conjugation of AFB₁-8,9-epoxide with GSH, it has been shown that the alpha class but not mu-class of GST possess greatest ability to metabolize activated AFB₁. Although the rat pi-class GST cannot catalyze this reaction it might be expected that the theta-class enzyme GST is active towards AFB₁-8,9-epoxide. By contrast with the rat, the mouse exhibits high constitutive levels of GST activity towards AFB₁-8,9-epoxide and alpha-class GST in Swiss-Webster mice possess high activity towards AFB₁-8,9-epoxide and can protect against DNA-binding by AFB₁ metabolites. Neither the murine mu-class nor pi-class GST can detoxify activated AFB₁ and all the activity towards this substrate is contributed by the alpha-class GST. It can be concluded that in the mouse the theta-class enzymes do not play a major role in the detoxification of activated AFB₁. Hamster liver contains significant levels of AFB₁-GSH-conjugating activity but the GST involved have not been characterized. In human liver, GST does not appear to play as important a role in providing protection against AFB₁ as the rodent GST. The *in vitro* studies have suggested that in comparison with rodents, relatively little AFB₁-GSH conjugate is produced by human liver, but insufficient data exist to be certain that this reaction is not of physiological importance in man, particularly as an aflatoxin mercapturate has been detected in the urine of marmoset monkeys treated with AFB₁. The ability of human alpha-class

GST to detoxify activated AFB₁ has not been examined systematically. Three separate alpha-class isoenzymes, which represent the dimeric combinations of two distinct subunits (B1 & B2) have been described in human liver. Furthermore, it is not known whether man possesses inducible GST and if so, whether these might be involved in AFB₁ metabolism [34]. Nevertheless, it has been shown that in the humans, the GST with the highest activity toward AFB₁ exo-epoxide is the polymorphic hGSTM1-1 which is absent in about 50% of individuals in most human populations. This suggests that AFB₁-epoxide individuals lacking the beneficial effects of hGSTM1-1 may be at elevated risk. Indeed some reports suggest that the GSTM1 genetic polymorphism may affect AFB₁ detoxification in human liver. In contrast to the liver, the lung is composed of many different cell types and expression of GSTs in different human lung cell types is heterogenous. Thus certain cell types with low levels of GSTs or lacking specific GST isoforms may be at higher risk of AFB₁ toxicity [72]. GSTP was also demonstrated to significantly increase in early hepatocarcinogenesis and hepatocellular carcinoma compared to their adjacent normal tissues. Loss of GSTP1 has been suggested to increase the risk of DNA damage and mutation. Moreover, up-expression of GSTA was suggested to protect liver cells against oxidative stress via an extracellular signal-regulated kinases (ERKs) and p38 kinase (p38K)-related pathway, as well as through the inhibition of H₂O₂-induced apoptosis to inhibit reactive oxygen species (ROS)- induced lipid peroxidation. It was suggested that inactivated or down-regulated GSTP1 and GSTA1 genes could increase genomic damage when individuals were exposed to carcinogens. [14]. GSTs have also been shown to exhibit GSH-dependent peroxidase activity and thus may be involved in resistance to oxidative stress. Cytosolic GSTs have been identified in almost all organisms, with mammalian GSTs the most clearly characterized [Burns et al. 2005].

Besides the formation of GSH conjugates, glucuronide and sulfate conjugates of AFB₁ have also been described in a variety of species including rat, mouse, monkey and trout. The ability to form these alternative secondary metabolites may be of considerable physiological importance in species, like the trout, that are unable to produce AFB₁-GSH conjugates. Before AFB₁ can form glucuronide and sulfate conjugates it requires to be hydroxylated. The primary metabolites AFM₁, AFP₁, and AFQ₁ can readily form glucuronide or sulfate conjugates. Whilst such conjugation reactions may aid excretion of aflatoxin, their toxicological value is unclear as such hydroxylated metabolites are not particularly harmful because they are not subject to 8,9-epoxidation. However, it has been proposed that AFB₁ is itself capable of forming glucuronide and sulfate conjugate; these reactions might entail a molecular rearrangement possibly involving the addition of water to the keto group in the cyclopentone ring, that result in the introduction of a hydroxyl group into the AFB₁ structure. This proposal is of particular interest as it enables the direct detoxification of AFB₁ through reactions that may not involve cytochrome P450. These workers have also proposed that amines, thiols and alcohols might also be conjugated to AFB₁ via the keto group in the cyclopentone ring [34].

Alternatively, the AFB₁-epoxide can hydrolyse spontaneously to AFB₁-dihydrodiol. This is not a true detoxification process as the dihydrodiol product can rearrange at neutral pH values to form a dialdehydic phenolate ion. This AFB₁-dialdehyde can undergo Schiff-base formation with primary amine groups in proteins and is therefore likely to be cytotoxic.

Recently, a novel AFB₁-aldehyde reductase (AFB₁-AR) purified from ethoxyquin (EQ)-treated rat liver has been shown to metabolize the dialdehyde form of AFB₁-dihydrodiol to an AFB₁-dialcohol and its relative importance in AFB₁ detoxification may be considerable [35]. The toxicity of AFB₁ is selective towards certain species. In contrast with the mouse and hamster, the rat, guinea pig and man are susceptible to the hepatotoxic effects of AFB₁ [34]. The toxicity of the mycotoxin is based on a balance between the rate of primary activation of AFB₁ and the rate of detoxification of primary metabolites or repair of cellular damage, determined by the relative activity of enzymes responsible for these reactions; the differential toxicity of AFB₁, between species is thought to be due mainly to different levels of activity of xenobiotic-metabolizing enzymes. In this regard, the livers of mice which are resistant to the hepatotoxic effects of AFB₁ contain high concentrations of a Yc-type GST subunit [55] that has considerable GSH conjugating activity towards AFB₁-epoxide [34], [37], [10], [9]. By contrast, the Fischer rat, an inbred strain that is five times more susceptible to AFB₁-induced liver cancer than the Wistar rat [34], possesses 20-fold less hepatic AFB₁-GSH-conjugating activity than the mouse. Fischer rats can, however, be protected against AFB₁ by treatment with the antioxidant EQ. It has shown that following EQ-treatment the livers of Fischer rats express a GST subunit that is immunochemically related to the mouse Yc subunit [35]. Moreover, this inducible polypeptide (Yc2, subunit 10) has high activity towards AFB₁-epoxide [35]. Thus, the Yc2 subunit is thought to confer protection against AFB₁, and its induction by EQ is likely to be one of the key mechanisms for the protective action of this anti-carcinogen [56].

The transport of foreign compounds out of cells can be achieved by at least two distinct families of efflux pump, both of which may provide protection against AFB₁ by helping eliminate the mycotoxin from target cells. The best characterized of these two pumps is P-glycoprotein, the product of the *mdr 1* gene which has been studied extensively because of its involvement in acquired resistance to anticancer drugs. The other pump is the glutathione S-conjugate carrier which is responsible for the transport of endogenous compounds such as oxidized glutathione and leukotriene C₄ as well as glutathione conjugates of foreign compounds an example of which might be S-(2,4-dinitrophenyl)glutathione. Both pump systems are ATP-dependent and are inhibited by vanadate but differ in that P-glycoprotein appears to have specificity towards hydrophobic compounds whereas the glutathione S-conjugate carrier is as its name suggests specific for leukotrienes and drug-glutathione conjugates. Although it is not known whether P-glycoprotein is able to transport AFB₁ the broad specificity of this efflux pump and its activity towards hydrophobic drugs suggests that this is likely. It also appears highly probable that the glutathione S-conjugate carrier is responsible for the transport of AFB₁ conjugated with GSH. Both P-glycoprotein and the glutathione S-conjugate carrier are expressed in the liver which is compatible with the hypothesis that these pumps could be involved in the efflux of AFB₁ and its metabolites. The involvement of P-glycoprotein in AFB₁ transport is supported by the fact that aflatoxin has been shown to induce mRNA encoding this protein in mouse liver. [36].

Relatively little is known about the enzymes responsible for the removal of AFB₁ that is bound covalently to DNA in mammalian cells. Exposure of cells to AFB₁ results in the formation of three major adducts. Of these, trans-2,3-dihydro-2-(N⁷-guanyl)-3-hydroxy AFB₁

(AFB₁-N⁷G) is the most abundant. It is chemically unstable and is lost spontaneously from DNA *in vitro* to yield apurinic sites. The other two adducts, 2,3-dihydro-2-(N-formyl-2,3,6-triamino-4-oxypyrimidine-N-yl)-3-hydroxy AFB₁ and 8,9-dihydro-8-(2-amino-6-formamide-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB₁ (AFB₁-FAPY and AFB₁III respectively) are not spontaneously but appear to be removed catalytically by DNA repair enzymes. The loss of AFB₁-DNA adducts *in vivo* is biphasic and this occurs through two distinct mechanisms. Following exposure to AFB₁, all adduct species are removed rapidly until less than 1000 adducts per cell remain. Once this point is reached the AFB₁-FAPY and AFB₁III adducts are no longer removed and only AFB₁-N⁷G is lost but at a much slower rate from the cell [36].

3.4.4. Conclusion and future directions

Evidences presented in this review article clearly show that glutathione conjugation to aflatoxin metabolites which has been detected in aflatoxin-producing fungi as well as liver tissues of mammals play a crucial role in reducing the interaction of aflatoxins with cellular macromolecules. However further studies is needed to answer the main questions about the contribution of glutathione conjugation system in removing aflatoxin in different cellular systems. The future direction of this topic is to find out experimental-based answers to the following questions:

1. What is the relationship between the rate of aflatoxin metabolism and the level of aflatoxin-GSH conjugate formation?
2. Which classes of glutathione S-transferases in each cellular system is directly responsible for involvement of aflatoxin-GSH conjugate formation
3. What is the relationship between the efficiency of glutathione conjugation system and toxic action of aflatoxins in different cell systems.

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